as follows:

F1

--1. (2X amended) A protein [comprising] consisting of a CRAF1 amino acid sequence shown in Figure 1 truncated at the amino terminus by [at least] 323 amino acid residues up to 414 amino acid residues, or a variant thereof wherein the variant comprises a conservative amino acid substitution, capable of inhibiting CD40-mediated cell activation.--

**REMARKS**

Claims 1-20 were pending. The Examiner withdrew claims 5-20 from further consideration. Applicants have canceled claim 2 without prejudice. Applicants have amended claim 1 to more particularly point out the presently claimed invention. Support for the amendment may be found in Figure 1 and inter alia in the specification, for example on page 8, lines 3-8. Support for "conservative amino acid substitution" may be found on page 9 in Table 1 of the subject specification. Applicants have amended the specification to include the appropriate Sequence ID Number as requested by the Examiner. Applicants maintain that these amendments raise no issue of new matter. Thus, claims 1, 3 and 4 are pending.

**Election/Restriction**

The Examiner stated that applicant's election with traverse of Group I, claims 1-4 in Paper No. 11 is acknowledged. The Examiner stated that the traversal is on ground(s) that the process of Group II cannot be practiced with another materially different product, as now amended. The Examiner did not find this persuasive because, as made of record in the Restriction of February 28, 1998, the inventions can allegedly be shown to be

distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product, or (2) the product as claimed can be used in a materially different process of using that product (MPEP §806.05(h)). The Examiner stated that in the instant case, the polypeptide of Group I may be used in screening assays or to generate antibodies. The Examiner stated that the requirement is still deemed proper and is therefore made final. The Examiner withdrew claims 5-20 from further consideration and stated that claims 1-4 are under consideration in the application.

In response, applicants acknowledge that the Examiner withdrew claims 5-20 from consideration.

**Specification**

The Examiner objected to the disclosure because the specification at page 22 contains a sequence disclosure without it's corresponding SEQ ID No. The Examiner required appropriate correction.

In response, applicants have amended the disclosure to include the SEQ ID NO where appropriate on page 22. In view of this amendment, applicants request that the Examiner reconsider and withdraw this objection in view of the amendment.

**Rejection Under 35 U.S.C. §112, second paragraph**

On page 3 of the August 18, 1998 Office Action, the Examiner rejected claims 1-4 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner stated that the claims are indefinite in that they

only describe the protein of interest by an arbitrary name. The Examiner stated that while the name itself may have some notion of the activity and function of the protein, there is nothing in the claims which distinctly and definitely describes or points out the protein. The Examiner stated that others in the field may isolate the same CRAF1 protein and give such an entirely different name. The Examiner stated that applicant should particularly point out and distinctly claim the CRAF1 protein by claiming characteristics associated with the protein. The Examiner stated that claiming biochemical molecules by a particular name given to the protein by various workers in the field fails to distinctly point out what the protein is. The Examiner also noted that the instant recitation of an apparently truncated protein by the open language "protein comprising CRAF1 truncated..." is confusing because it isn't clear if more than the truncated protein is contemplated.

The Examiner also stated that the phrase "CD40 mediated cell activation" found in claim 1 is vague and indefinite because the metes and bounds of what is including and definitive of "activation" cannot be determined. The Examiner stated that neither the claims nor the specification provide a clear definition of what parameters determine or are definitive of cell activation. The Examiner also stated that dependent claims 2-4 do not clarify the above indefiniteness.

In response, applicants respectfully traverse the rejection of claims 1-4 under 35 U.S.C. §112, second paragraph. Without conceding the correctness of the Examiner's position, applicants have amended claim 1 hereinabove and maintain that the amendment raises no issue of new matter. Applicants have amended the claim to recite "consisting of" in order to address and obviate the Examiner's objection to the language "comprising." Applicants have also amended claim 1 to include a reference to the amino acid sequence of CRAF1 shown in Figure 1 of the present

application. Applicants maintain that the presently claimed invention is particularly pointed out in the language of claim 1. Applicants maintain that the present invention is distinctly claimed and request the Examiner to reconsider and withdraw this ground of rejection.

As to the Examiner's concern regarding the phrase "CD40 mediated cell activation," applicants maintain that one of skill in the art would know how to determine cell "activation" and to illustrate this point, applicants have attached hereto as **Exhibit 1** a reference which indicates that one of skill would know the metes and bounds of CD40 mediated cell activation prior to the effective filing date of March 11, 1996. The reference, Potocnik et al., (1990) "Expression of Activation Antigens on T Cells in Rheumatoid Arthritis Patients" Scand. J. Immunol. 31:213-224, examined both normal and rheumatoid patient synovial tissue for CD40 expression by the monoclonal antibody B-E10. CD40 triggering of B cells was known prior to the effective filing date of the present application. CD23 upregulation is one marker of B cell activation.

In addition, applicants draw the Examiner's attention to page 24, beginning at line 24 of the present specification. Therein, applicants describe the upregulation of CD23 as an indicator of cell activation in response to CD40 triggering. In this working example, a Ramos cell line transfected with the C26 clone had diminshed capacity to upregulate CD23 in response to CD40L-CD40 signals. Thus, the line transfected with the C26 clone demonstrated inhibition of CD40 mediated cell activation.

Furthermore, applicants draw the Examiner's attention to Hu et al. J. Biol. Chem., 269(48):30069. This reference is dated December 2, 1994 on its face and clearly indicates that one of skill would have understood the metes and bounds of the phrase "CD40 mediated cell activation." In the introductory paragraph,

Hu et al. disclose that "CD40 activation is critical for B-cell proliferation, immunoglobulin class switching, and rescue of germinal center B-cells from apoptosis following somatic mutation." Clearly, there are numerous markers of CD40 mediated cell activation which would have been known to one of skill in the art which mark the activation of a cell due to CD40 signalling.

In view of the above remarks and amendments, applicants respectfully request the Examiner reconsider and withdraw this ground of rejection.

**Rejection Under 35 U.S.C. §112, first paragraph - Written Description**

The Examiner rejected claims 1-4 as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Examiner stated that claim one (and therefore dependent claims 2-4) has been amended to recite truncation of "at least 323 amino acid residues up to 414 amino acid residues" for which the specification fails to provide support. The Examiner stated that the specification provides support for truncations of about 323 amino acids to about 414 amino acids. The Examiner further asserted that nowhere is it set forth that the truncation must be at least 323 amino acids. The Examiner stated that the original language indicates that the exact value may vary. Similarly, the Examiner stated, the specification provides support for truncations of about 414 amino acids, not exactly 414 amino acids, the original specification and claim language indicates that the truncation may be slightly more than 414 amino acids, but must be about 414 amino acids. Thus, the Examiner

stated, the instant amendment alters the claimed invention in scope from that originally disclosed. The Examiner suggested that in order to distinctly claim the truncation as supported by the original disclosure, without introducing indefiniteness, the claims may be amended to recite amino terminus truncations of 323 amino acids to 414 amino acids residues which is contemplated by the original specification, or more ideally, to recite the amino acid range of the truncated protein as disclosed on page 5 and in Figure 2.

In response, applicants respectfully traverse the rejection of claims 1-4 under 35 U.S.C. §112, first paragraph. Without conceding the correctness of the Examiner's position, applicants have amended claim 1 to more particularly point out the presently claimed invention. Applicants have removed the phrase "at least" from claim 1. The presently claimed invention is directed to a protein consisting of a CRAF1 amino acid sequence shown in Figure 1 truncated at the amino terminus by 323 amino acid residues up to 414 amino acid residues, or a variant thereof wherein the variant comprises a conservative amino acid substitution, capable of inhibiting CD40-mediated cell activation. Applicants point out that the sequence claimed in claim 1 is supported by the subject specification, specifically, Figure 1. Also, applicants have described such proteins as presently claimed in the subject specification on page 8, line 3 to page 12, line 14 and have provided examples of such a protein in Figures, 1, 2A and 3 and on pages 5, lines 3-13 and page 5, line 31 to page 6, line 28. The specification provides a full written description of the claimed proteins and includes several working examples. See, for example, the C26 clone shown in Figure 3. Thus, applicants maintain that the specification provides sufficient written description of the presently claimed invention. In view of the above remarks and amendments, applicants respectfully request the Examiner to reconsider and withdraw this ground of rejection.

**Rejection Under 35 U.S.C. §112, first paragraph - Enablement**

The Examiner rejected claims 1-4 under 35 U.S.C. §112, first paragraph as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected to make and/or use the invention. The Examiner stated that the specification is not enabling for the invention as broadly claimed. The Examiner stated that the specification is not enabling for any variant of truncated CRAF1 protein or truncated CRAF1 proteins which inhibit any CD40 mediated cell activation.

The Examiner stated that the factors to be considered have been summarized as the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art and the breadth of the claims. Ex Parte Forman, (230 USPQ 546 (Bd Pat. App. & Int. 1986)).

The Examiner stated that the instant specification maps the regions responsible for binding to CD40 to the TRAF domain while the function of the other domains present in CRAF1 remains speculative. Further, the Examiner stated that the specification discloses that truncated CRAF1 proteins retaining the C-terminal TRAF domain (TRAF-C domain) defined by amino acid residues 415-567 of SEQ ID NO: 1 or 2 function as individual CD40 binding units which may have inhibitory properties. The Examiner stated that the specification further discloses that one specific truncated protein, C26, which consists of amino acid residues 324-567, serves as a dominant negative protein, inhibiting CD40 cell mediated CD23 upregulation.

The Examiner stated that thus, the disclosure of the instant specification supplies sufficient objective evidence and guidance to make it predictable that amino truncated CRAF1 proteins retaining TRAF-C domain would bind to CD40 with properties similar to the C26 example. However, the Examiner stated, the specification does provide sufficient guidance and objective evidence that any variant of a CRAF1 truncated protein would reasonably be expected to retain CD40 binding properties and inhibitory activity. The Examiner stated that the specification contemplates variants including amino acid substitutions, deletions, or insertions, or other chemical modifications of substituents (pages 8-11). However, the Examiner stated, the specification fails to provide sufficient guidance (with the exception of conservative substitutions as recited in claim 2) directing one of skill to determine where within the truncated protein the modifications are acceptable and what types of modifications are predicted to result in similar binding and inhibitory activities. The Examiner stated that the specification discloses only that the TRAF-C domain is necessary, but supplies insufficient information regarding what sequences within this domain may be modified and still predictably result in similar activity. The Examiner stated that the amino acid sequence of a protein determines its structural and functional properties, and predictability of which amino acids can be substituted or modified within a protein's sequence and still result in similar activity is extremely complex, and well outside the realm of routine experimentation, because accurate predictions of a protein's structure and function from mere sequence data are limited. The Examiner stated that since detailed information regarding the structural and functional requirements of this protein are lacking, it is unpredictable as to which amino acid substitutions, if any, meet the limitations of the claim. The Examiner stated that furthermore, while recombinant techniques are available, it is not routine in the art to screen large numbers of substituted proteins where the

expectation of obtaining similar activity is unpredictable based on the instant disclosure. Therefore, the Examiner stated, one of ordinary skill would require guidance, such as information regarding the extent of substitution and the location and the specific amino acid changes which would result in the preservation of the stated activity. The Examiner stated that therefore, it would require undue experimentation by one of skill in the art to practice the invention as claimed without further guidance from the instant specification.

The Examiner stated that additionally, as discussed supra, the phrase "CD40 mediated cell activation" is vague and indefinite because it is not clear what measurable properties of "activation" correlate with CD40 mediated cell "activation," nor is it clear when a cell is determined to be "activated." The Examiner stated that the specification provides guidance for the determination of inhibition by detecting CD40 mediated CD23 upregulation. The Examiner stated that there is insufficient guidance or objective evidence to support the correlation of any other measurable "properties" and cell "activation." The Examiner stated that the specification contemplates that "activation" may include any and all intracellular signalling, immune responses, allergic responses, apoptosis (pages 14-18), yet there is no guidance provided teaching the accurate determination and measurement of "activation" of these processes and their inhibition. The Examiner stated that absent further guidance it would require undue experimentation to practice the instant invention and to predictably identify inhibitory truncated proteins as broadly claimed.

In response, applicants respectfully traverse the rejection of claims 1-4 under 35 U.S.C. §112, first paragraph and maintain that the specification fully enables the presently claimed invention. Guidance as to where within the truncated protein the variation from the CRAF1 sequence may be found in the

specification on page 8, beginning at line 19. Conservative substitutions are fully described and enabled. Table 1, on page 9 of the specification also provides specific guidance as to which substitutions would be considered conservative. Applicants maintain that the specification and the references included therein (i.e., Dayhoff in the Atlas of Protein Sequence and Structure (1988) as recited on page 9 of the specification) provides a fully enabling disclosure for the presently claimed invention.

As to the Examiner's comments regarding "CD40 mediated cell activation," applicants respectfully traverse. Applicants point out that the present invention is directed to a protein consisting of a CRAF1 amino acid sequence shown in Figure 1 truncated at the amino terminus by 323 amino acid residues up to 414 amino acid residues, or a variant thereof wherein the variant comprises a conservative amino acid substitution, capable of inhibiting CD40-mediated cell activation. Applicants maintain that the specification fully enables CD40 mediated cell activation and that one of skill in the art would know how to determine CD40 mediated cell activation.

First, the specification provides a working example of determining CD40 mediated cell activation. The specification shows the determination of the level of CD23 expression and discloses that a lack of upregulation of CD23 in a Ramos cell line indicates an inhibition of CD40-mediated cell activation. Thus, the cell activation was measured by determining the level of CD23 upregulation. This is a marker that one can use to determine CD40-mediated cell activation.

Second, applicants maintain that one of skill in the art would know other markers of CD40-mediated cell activation and would know how to measure them. See for example, Exhibit 1 attached hereto. Applicants refer the Examiner to the discussion of

Exhibit 1 hereinabove and maintain that Potocnik et al. (1990) disclose differences in cell surface marker expression between T cells from normal individuals and T cells recovered from inflamed joints of rheumatoid arthritis (RA) patients (activated T cells). Potocnik et al. found the molecules B-C5, CD39, CD40, CD45 RO, CD54, CD76 and potentially 1D11 to be substantially upregulated on T cells from various body compartments in RA patients. Clearly, as of 1990, one of skill in the art would have known of these activation markers and how to measure them.

In view of the amendments and remarks, applicants respectfully request the Examiner to reconsider and withdraw this ground of rejection.

**Rejection Under 35 U.S.C. §102(b)**

The Examiner rejected claims 1-4 under 35 U.S.C. §102(b) as being anticipated by Sato et al. (FEBS Lett. 358:113-118, Jan. 23, 1995) or Hu et al. (J. Biol. Chem. 269:30069-30072, Dec. 1994 - IDS) or Cheng et al. (Science 267:11494-1498, March 10, 1995 - IDS).

The Examiner stated that Sato et al. teach a truncated clone of CAP-1 which encodes little more than the C-terminal region of CAP-1, between amino acid residues 384-540 sufficient to mediate binding to CD40. The Examiner stated that Sato et al. are silent regarding the inhibitory properties of the truncated protein, however, CAP1 is identical to the instant CRAF1, and the capability of the truncated protein to inhibit CD40 mediated activation events such as CD23 would be an inherent property of the truncated product.

The Examiner further stated that Hu et al. teach the same CAP1, CAP1, CRAF1 protein, termed CD40bp and a truncated version of only the C-terminal half, from amino acid residue 297-the end,

which binds to CD40. The Examiner stated that like Sato et al., Hu et al. is silent regarding the inhibitory properties of the truncated protein, however, such properties would be inherent to the product.

The Examiner stated that Cheng et al. teach that truncated CRAF1, clone C26, identical to the instant product, inhibits CD40-mediated up-regulation of CD23.

The Examiner noted that the proteins designated CRAF1, CAP1 (Sato et al.), CD40bp (Hu et al.), as well as LAP1 (Mosialos et al. - IDS) refer to the same protein, as acknowledged in the instant specification on page 2 and as exemplified by the enclosed sequence data.

The Examiner stated that thus, each of Sato et al., Hu et al., and Cheng et al. teach truncated CRAF1 protein products as claimed which would inherently possess the same inhibitory properties.

In response, applicants respectfully traverse the rejection of claims 1-4 under 35 U.S.C. §102(b) over Sato et al., Hu et al. or Cheng et al. Applicants maintain that none of these references anticipate the presently claimed invention.

First, applicants maintain that Cheng et al. is not a proper reference under 35 U.S.C. §102(b). The present application claims the priority of U.S. Provisional Application No. 60/013,199, filed March 11, 1996. As to the Cheng et al. reference, the journal Science, volume 267 dated March 10, 1995, was placed into the U.S. Mail on March 10, 1995. Applicants attach hereto as **Exhibit 2** a letter from Ms. Helen Williams of the American Association for the Advancement of Science indicating that March 10, 1995 issue date of Science was in fact the date of mailing. Thus, subscribers to the journal Science

would not have been in possession of the journal dated March 10, 1995 until, at the earliest, March 11, 1995. Furthermore, March 11, 1995 is not more than one year prior to March 11, 1996. Therefore, applicants maintain that Cheng et al. is not a proper reference under 35 U.S.C. §102(b). Accordingly, applicants respectfully request the Examiner to reconsider and withdraw this ground of rejection based upon Cheng et al.

Furthermore, if the Examiner were to reject claims 1, 3 and 4 under 35 U.S.C. §102(a) over the Cheng et al. reference, applicants maintain that the Cheng et al. reference is not a publication "by others" as required under 35 U.S.C. §102(a). If the Examiner rejects the pending claims under 35 U.S.C. §102(a) in view of Cheng et al., applicants are prepared to submit a Declaration Under 35 U.S.C. §1.132 as evidence that David Hong (the only author of Cheng et al. who is not named as an inventor of the present application) did not contribute to the conception of the claimed invention.

Applicants maintain that neither Sato et al. nor Hu et al. anticipate the presently claimed invention. In order for a reference to anticipate the claimed invention, it must disclose every element of the claimed invention. Sato et al. do not disclose the truncated protein as presently claimed. The Examiner stated that "CAP1 is identical to the instant CRAF1" on page 8 of the August 18, 1998 Office Action. Applicants respectfully disagree. Applicants point out that the CAP1 sequence disclosed by Sato et al. is a different length than the CRAF1 sequence shown in Figure 1 of the present application. Furthermore, there are numerous amino acid sequence differences between the two proteins. Finally, Sato et al. do not disclose a truncated protein as presently claimed. It is clear that the truncated protein as presently claimed is different than the CAP1 sequence disclosed in Sato et al. Since the proteins are different on their face (see Figure 2 of Sato et al. as compared

with Figure 1 of the present specification), truncated versions of each protein would also be different. The inherency argument presented by the Examiner does not hold true. Clearly, different proteins will have different inherent characteristics. Therefore, Sato et al. do not anticipate the presently claimed invention.

Hu et al. do not anticipate the presently claimed invention. Hu et al. do not disclose the truncated protein presently claimed. Figure 4, panel A of Hu et al. discloses an amino acid sequence of a protein termed CD40bp. Hu et al. do not disclose a protein consisting of a CRAF1 amino acid sequence shown in Figure 1 of the subject specification which is truncated at the amino terminus by 323 amino acid residues up to 414 amino acid residues, or a variant thereof wherein the variant comprises a conservative amino acid substitution, capable of inhibiting CD40-mediated cell activation. The Examiner stated that Hu et al. teaches "truncated version of only the C-terminal half, from amino acid 297-the end...." Applicants disagree with the Examiner's summary of the Hu et al. disclosure and maintain that Hu et al. do <u>not</u> teach a truncated version of CD40bp. Hu et al. merely disclose various characteristics of the <u>full-length</u> protein (see column 1, page 30072) and do not disclose a truncated protein. However, Hu et al. do mention a truncated TRAF2 protein, which is missing the RING finger domain, in the first sentence of the last paragraph of the reference. This is not a disclosure of a truncated protein as presently claimed. Applicants also point out that the Hu et al. reference does not disclose such a truncated protein as presently claimed which is capable of inhibiting CD40-mediated cell activation.

Thus, applicants maintain that the presently claimed invention is not anticipated by either Sato et al. or Hu et al. Furthermore, applicants maintain that Cheng et al. is not a proper reference under 35 U.S.C. §102(b). In view of the above

amendments and discussion, applicants respectfully request that the Examiner reconsider and withdraw the outstanding grounds for rejection and earnestly solicit the allowance of pending claims 1, 3 and 4.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the $435.00 extension of time fee, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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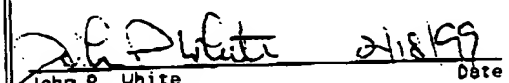
I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents Washington, D.C. 20231.



2/18/99
Date

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*Scand. J. Immunol.* 31, 213–224, 1990

# Expression of Activation Antigens on T Cells in Rheumatoid Arthritis Patients

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Potocnik, A.J., Kinne, R., Menninger, H., Zacher, J., Emmrich, F. & Kroczek, R.A. Expression of Activation Antigens on T Cells in Rheumatoid Arthritis Patients. *Scand. J. Immunol.* 31, 213–224, 1990

The aim of our study was to identify differences in cell surface marker expression between T cells taken from the peripheral blood (PB) of healthy individuals and T cells recovered from inflamed joints of rheumatoid arthritis (RA) patients. Out of 118 monoclonal antibodies (MoAbs) directed against activation antigens on haematopoietic cells, 12 MoAbs recognizing nine distinct surface molecules were selected after a screening procedure to study the expression of the corresponding antigens on T cells from the PB, synovial fluid and synovial tissue of RA patients, and also on T cells from PB and spleens of controls. Using two-colour flow cytometry and immunohistology we found the molecules B-C5, CD39, CD40, CD45 R0, CD54, CD76 and potentially 1D11 to be substantially up-regulated on T cells from various body compartments in RA patients. We thus could determine that the cell surface of T cells in RA patients not only differs in MHC class II expression, but also in a number of other activation-associated cell surface molecules from T cells in healthy individuals.

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Lymphoid cells seem to play a major role in the pathogenesis and perpetuation of rheumatoid arthritis (RA). The majority of mononuclear cells found in synovial tissue (ST) and synovial fluid (SF) of inflamed joints are T lymphocytes [1, 3]. These T cells differ in a number of cell surface markers from T cells present in the peripheral blood (PB) of healthy individuals. The most conspicuous finding in this respect is a clearly elevated expression of MHC class II antigens on T cells in the PB and in particular in the SF and ST of patients with RA [7, 10, 15, 23]. This phenomenon is widely regarded as a sign of T-cell activation. Supporting this view are observations of a discretely increased spontaneous proliferation rate of T cells recovered from SF [21, 37]. On the other hand, the expression of other activation markers such as receptors for interleukin-2 or transferrin is in most cases barely up-regulated [6, 15, 22]. In contrast to in vitro-activated normal lymphocytes, MHC class II-bearing T cells from SF do not show signs of blastoid transformation [7]. Altogether these findings indicate that RA T cells share MHC class II expression with in vitro-activated PB T cells but clearly differ in surface phenotype and function. The reason for this divergence is unknown. In the present study we have examined the expression of other activation markers on the surface of RA T cells in order to delineate further the functional state of these cells in vivo.

## MATERIALS AND METHODS

*Patients and controls.* Ten patients with RA according to the 1987 revised ARA criteria [2] and one patient with a reactive synovitis caused by a knee injury were included in this study. Clinical data for these patients are given in Table 1. All RA patients had acute synovitis with hydrops. ST was obtained from patients at synovectomy. Spleen tissue was removed from patients undergoing abdominal surgery. None of the spleen donors show signs of an acute inflammation.

TABLE I. Patient characteristics

| Patient | Sex | Age | Dur. | Diagn. | Treatment | | | RF | ES |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | NSAID | SAARD | Glucocorticoids | | |
| M.M. | F | 30 | 110 | RA | Diclofenac | — | — | + | 8 |
| H.F. | M | 49 | 62 | RA | Diclofenac | — | — | + | 8 |
| W.M. | F | 76 | 26 | RA | Ketoprofen | Aurothioglucose | Methylprednisolone | — | 6 |
| P.T. | F | 60 | 50 | RA | Diclofenac | Aurothioglucose | — | — | 4 |
| T.P. | F | 59 | 38 | RA | Ketoprofen | | — | + | 3 |
| S.M. | M | 56 | 158 | RA | Ketoprofen | Aurothioglucose | — | + | 3 |
| K.A. | M | 67 | 122 | RA | Diclofenac | — | Prednisolone | — | 2 |
| O.M. | M | 47 | 3 | ReaSyn | — | — | — | — | 1 |
| A.T. | F | 57 | 87 | RA | Diclofenac | — | — | + | 5 |
| G.E. | F | 67 | 132 | RA | Diclofenac | — | — | + | 7 |
| M.T. | M | 66 | 156 | RA | Diclofenac | — | — | + | 4 |

Age in number of years.
Dur., duration of disease in months.
Diagnosis: RA, rheumatoid arthritis; ReaSyn, reactive synovitis.
NSAID, non-steroidal anti-inflammatory drugs; SAARD, slow-acting anti-rheumatic drugs.
RF, rheumatoid factor.
ESR, erythrocyte sedimentation rate (mm/h).

*Cell isolation.* Mononuclear cells were separated from heparinized PB or SF on a Ficoll-metrizoate (Lymphoprep, Nycomed, Oslo, Norway) density gradient. Synovectomy tissue was digested with 2 mg/ml collagenase (Biochrom, Berlin, FRG) and 0.15 mg/ml DNAse (Paesel, Frankfurt, FRG) for 2 h at 37°C in Hanks' balanced salt solution (HSS) without $Ca^{2+}$, $Mg^{2+}$ (Biochrom, Berlin, FRG), and subsequently fractionated by centrifugation on discontinuous gradients consisting of 10%, 20%, 50% and 60% Percoll (Pharmacia, Freiburg, FRG) in HSS without $Ca^{2+}$, $Mg^{2+}$. The fraction enriched for T cells was removed from the interface between the 50% and 60% Percoll layers.

*Monoclonal antibodies.* The monoclonal antibodies (MoAbs) used in this study are listed in Table II.

*Immunofluorescence flow cytometry.* $2-2.5 \times 10^5$ cells were washed twice with PBS containing 5% FCS and 0.05% sodium azide and then incubated for 30 min at 4°C with unconjugated MoAbs. The cells were then washed and labelled with fluorescein isothiocyanate-conjugated polyclonal goat anti-mouse immunoglobulin serum (Medac, Hamburg, FRG). For two-colour immunofluorescence studies an additional washing step was performed followed by incubation with normal mouse serum in order to block non-specific binding. The cells were stained with a phycoerythrin-conjugated antibody and washed again twice. In all studies 20,000 live cells were analysed on an EPICS 753 fluorescence-activated cell sorter (Coulter Electronics, Hialeah, USA) using propidium iodide gating for exclusion of dead cells [33]. The threshold for positive staining was defined to maximally include 1.5% of the negative controls. Throughout the study an identical setting of the instrument (gain, laser output, photomultiplier voltage) was used.

*Histology.* All samples were frozen and stored at −70°C or in liquid nitrogen. Cryostat sections were fixed for 10 min in acetone. Immunohistology was performed on cryostat sections using the alkaline phosphatase antialkaline phosphatase (APAAP) technique [8]. After pre-incubation, MoAbs were added for 1 h in dilutions of 1:50 or 1:100 in Tris-buffered saline (TBS) with 1% bovine serum albumin (BSA). After

TABLE II. Monoclonal antibodies used in this study

| Antibody | Specificity | Reference |
|---|---|---|
| OKT11 | CD2 | 36 |
| OKT3 | CD3 | 32 |
| Leu 4 | CD3 | 25 |
| UCHT1 | CD3 | 4 |
| 91D6 | CD4 | 17 |
| Leu 2a | CD8 | 9 |
| 50H19 | CD9 | 27 |
| OKM1 | CD11b | 5 |
| α-Tac | CD25 | 34 |
| L62 | CD25 | 11 |
| 4B4 | CD29 | 29 |
| OKT28 | CD39 | 11 |
| A1 | CD39 | 11 |
| B-E10 | CD40 | 11 |
| A6 | CD45 R0 | 11 |
| 7F7 | CD54 | 11 |
| OKT27 | CD54 | 11 |
| MEM-75 | CD71 | 11 |
| HD66 | CD76 | 11 |
| L243 | MHC class II | 24 |
| TU66 | — | 11 |
| B-C5 | — | 11 |
| 22.2 | — | 11 |
| 1D11 | — | 11 |
| ACT35 | — | 11 |
| VLA-1 | — | 18 |
| UM4D4 | — | 19 |
| 361 | — | Unpublished |
| 534 | — | Unpublished |

| corticoids | RF | ESR |
|---|---|---|
| — | + | 82 |
| — | + | 80 |
| prednisolone | — | 65 |
| — | — | 48 |
| — | + | 38 |
| — | + | 36 |
| alone | — | 25 |
| — | — | 17 |
| — | + | 53 |
| — | + | 70 |
| — | + | 42 |

drugs.

sphatase (APAAP) tech-
n. MoAbs were added for
:00 in Tris-buffered saline
albumin (BSA). After a

dies used in this study

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| II | 24 |
| | 11 |
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| | 11 |
| | 11 |
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| | 19 |
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| | Unpublished |

washing step a F(ab)$_2$ goat anti-mouse antibody (Dianova, Hamburg, FRG) was applied for 1 h (1:20 in TBS/1% BSA). APAAP complex (Dianova) at a dilution of 1:100 (in TBS/1% BSA) was added for 30 min after repeated washing steps. The alkaline phosphatase was developed in a solution containing 3 mg naphthol-AS-MX-phosphate in 200 μl *N,N*-dimethylformamide, 10 mg Fast Red TR (Sigma, Deisenhofen, FRG), and 2.4 mg levamisole in 10 ml TBS, pH 8.6 for 30 min. All incubations were performed at room temperature. The sections were counterstained with Mayer's haematoxylin.

## RESULTS

### *Expression of activation antigens on PB T cells from healthy donors and RA patients*

A panel of 118 MoAbs [11] was screened on unseparated cells from three SF and three ST samples of RA patients by one-colour flow cytometry, and 48 MoAbs showed positive staining. The criterion for the next screening step was absent or at least clearly lower expression of the corresponding antigens on PB mononuclear cells from healthy donors; 12 MoAbs with the greatest difference in expression were selected for all subsequent studies. The 12 MoAbs were then used to analyse T cells from PB of healthy individuals and RA patients by two-colour flow cytometry. Also included in these studies was a panel of 'standard' MoAbs in order to better characterize the T cell populations examined. The antigens recognized by this 'standard' set were CD4 and CD8 as classical markers for functional T-cell subsets, the adhesion molecules CD2, CD11b and CD29, and the activation-associated

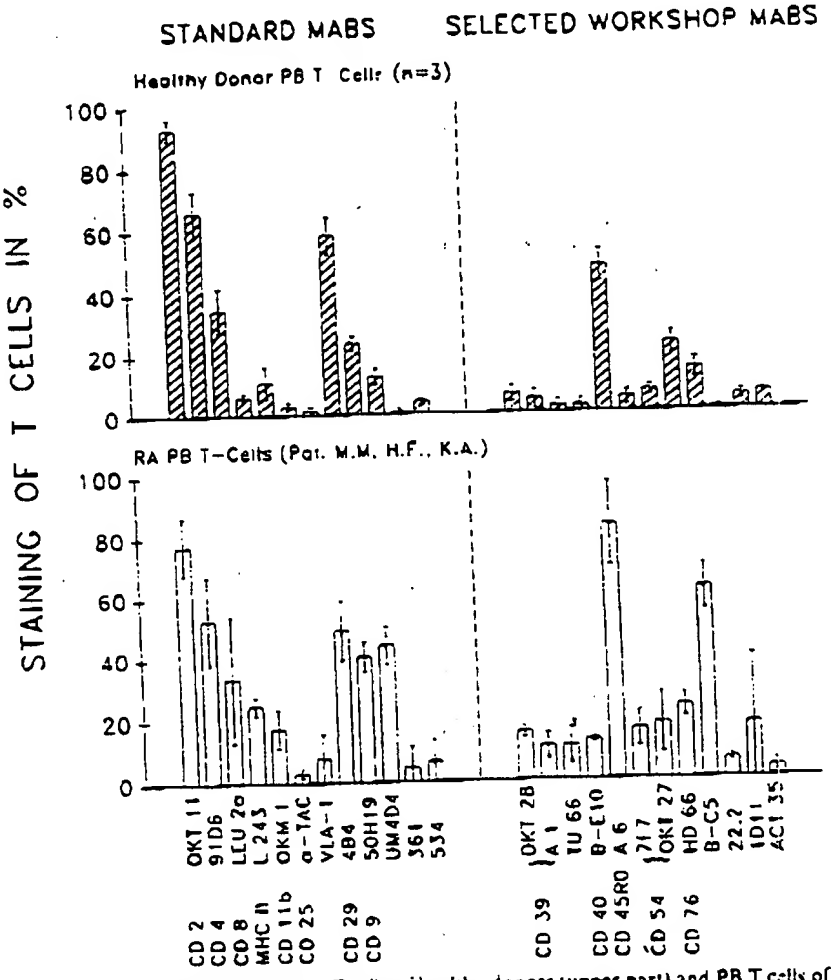


FIG. 1. Comparison between PB T cells of healthy donors (upper part) and PB T cells of RA patients (lower part) using two-colour flow cytometry. Vertical columns represent the mean percentages of staining of CD3$^+$ cells ± one standard deviation.

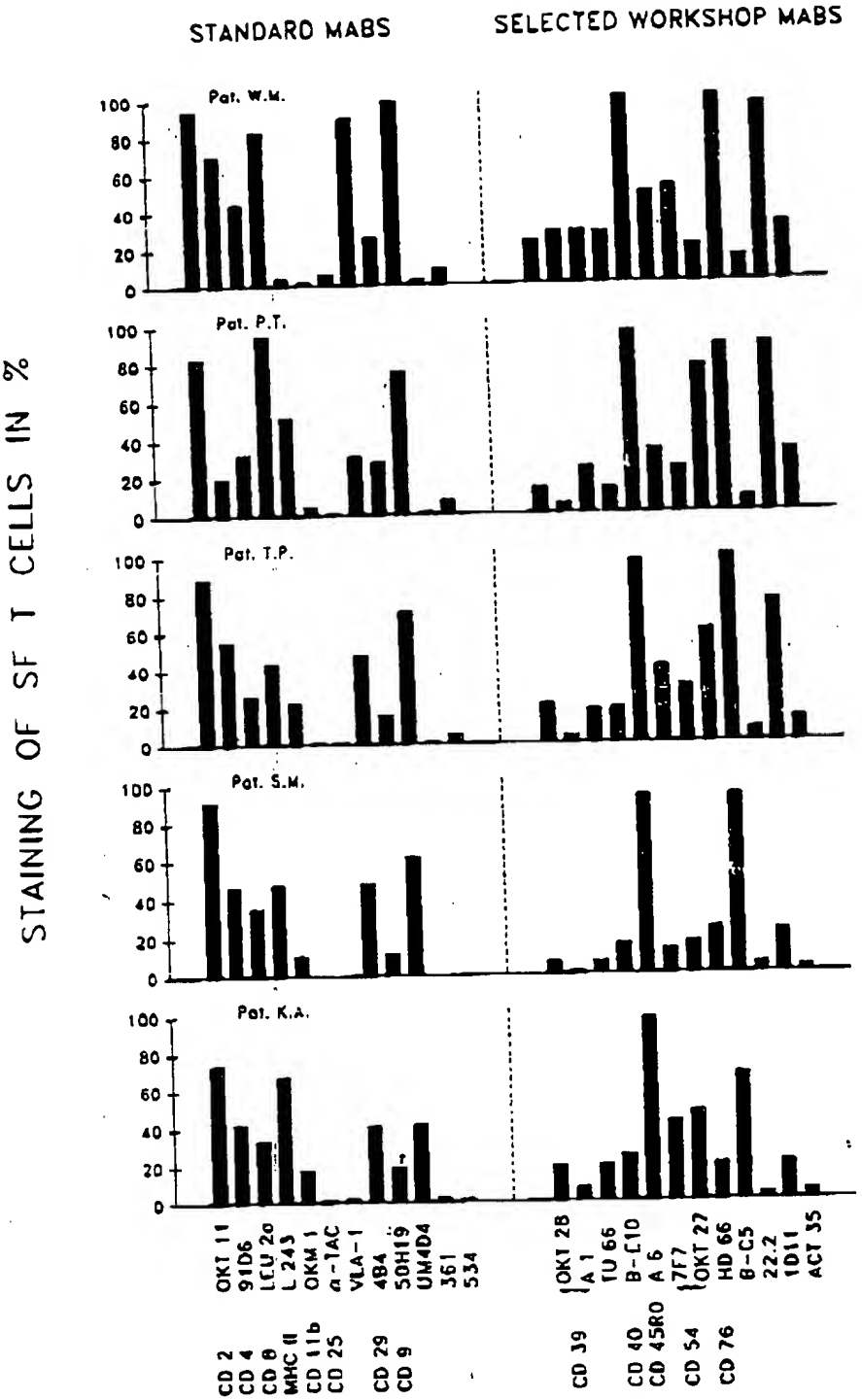


FIG. 2. Distribution of activation antigens on SF T cells of five RA patients. Vertical columns represent the percentages of staining of $CD3^+$ cells.

molecules MHC class II, CD25, VLA-1 [18], CD9 [20] and UM4D4 [19]. In addition, MoAbs 361 and 534 were used, since they recognize activation antigens on HUT 102 cells (O. Majdic, W. Knapp, personal communication). Fig. 1 shows PB T cells from healthy donors in comparison with PB T cells from RA patients stained with our standard set and the selected 12 MoAbs. On PB T cells from RA patients MHC class II products and the antigen recognized by UM4D4 showed elevated levels, which is in accordance with previously published data [12, 15, 19, 23]. In addition, the staining with MoAb 50H19 was clearly increased. In healthy individuals only three antigens (CD45 R0, CD76 and B-C5) out of the selected markers were present on resting PB T cells. In contrast, T cells from the PB of RA patients reproducibly showed a slightly elevated expression of most of the examined antigens. A particularly marked increase in staining was observed with the MoAbs A6 (CD45 R0) and B-C5, and to a lesser degree with the MoAbs 7F7/OKT27 recognizing the ICAM-1 molecule. Staining with MoAb 1D11 revealed a high variation of antigen expression on RA PB T cells.

*Expression of activation antigens on SF T-cells of RA patients*

As a next step SF T cells from RA patients were investigated by two-colour flow cytometry (Fig. 2). In general, SF T cells showed a higher expression of the examined activation antigens than PB T cells from RA patients. A constant feature in this respect was the very high percentage of MHC class II$^+$ and UM4D4$^+$ T cells in the standard panel. Another remarkable aspect was that the sum of CD4$^+$ and CD8$^+$ T cells in the SF was usually clearly lower than the number of cells bearing the CD3 molecule. In the panel of the selected workshop MoAbs we observed a further increased percentage of staining with the MoAbs A6 and B-C5, with almost all T cells expressing the corresponding antigens. A higher degree of staining was also found with MoAbs specific for the ICAM-1 (CD54) molecule. The antigens recognized by MoAbs HD66 (CD76) and 1D11 showed a high variability of expression on SF T cells.

*Direct comparison of activation on T cells from PB, SF and ST*

In one single patient we were able to compare the surface phenotype of T cells obtained from PB, SF and ST at the same time (Fig. 3; the results for SF T cells are also shown as SF Pat. K.A. in Fig. 2). Unless the expression of a given activation antigen was already strongly up-regulated

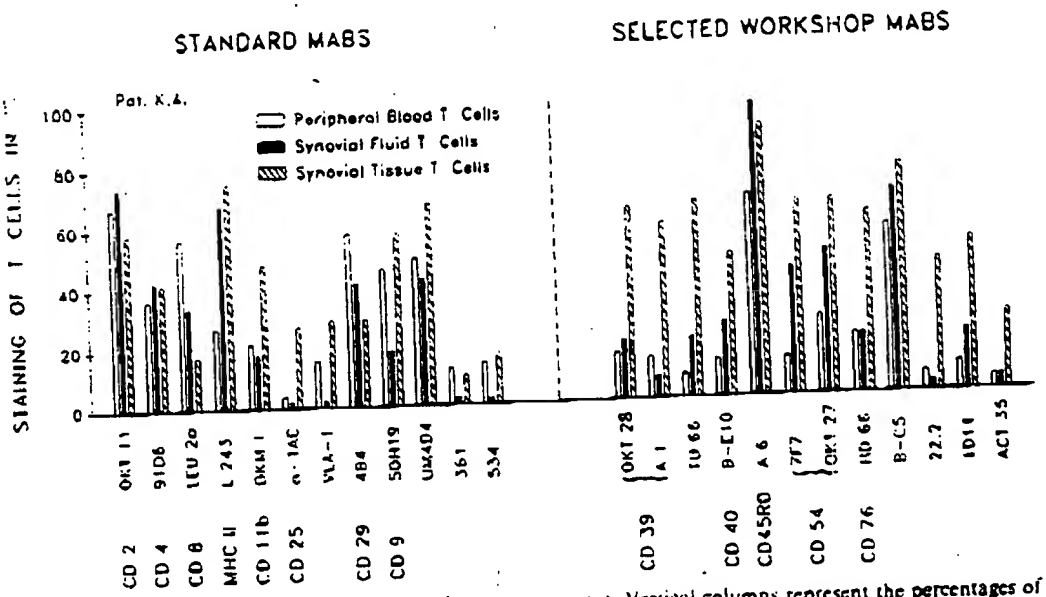


FIG. 3. Comparison of PB, SF and ST T cells of one single patient. Vertical columns represent the percentages of staining of CD3$^+$ cells.

TABLE III. Immunohistological staining patterns of antibodies directed against activation antigens on synovial tissue

| | | Synovial lining cells | | | | Endothelial cells | | | | Spindle-shaped cells | | | | Inflammatory infiltrates | | | | Lymphoid follicles | | | | PMN (vessels) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pat. | | | | Pat. | | | | Pat. | | | | Pat. | | | | Pat. | | | | Pat. | | |
| | | Control | 1 | 2 | 3 | Control | 1 | 2 | 3 | Control | 1 | 2 | 3 | Control | 1 | 2 | 3 | Control | 1 | 2 | 3 | Control | 1 | 2 | 3 |
| *Antibodies* | | | | | | | | | | | | | | | | | | | | | | | | | |
| UCHT1 | (CD3) | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | — | 1 | 2 | 2 | — | 4 | 4 | 3 | 0 | 0 | 0 | 0 |
| L62 | (CD25) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | 1 | 1 | — | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| MEM-75 | (CD71) | 4 | 3 | 2 | 3 | 4 | 0 | 0 | 3 | 1 | 2 | 2 | 3 | — | 3 | 2 | 3 | — | 1 | 1 | 3 | 0 | 0 | 0 | 0 |
| TU39 | (MHC-II) | 4 | 3 | 3 | 2 | 4 | 0 | 0 | 3 | 2 | 2 | 2 | 2 | — | 3 | 3 | 2 | — | 3 | 3 | 3 | 0 | 0 | 0 | 0 |
| OKT28 | (CD39) | 2 | 1 | 2 | 1 | 4 | 4 | 4 | 4 | 2 | 2 | 2 | 3 | — | 3 | 3 | 3 | — | 3 | 2 | 3 | 0 | 0 | 0 | 0 |
| A1 | (CD39) | 2 | 2 | 2 | 2 | 4 | 4 | 4 | 4 | 2 | 2 | 2 | 2 | — | 3 | 3 | 4 | — | 2 | 2 | 2 | 1 | 0 | 0 | 0 |
| TU66 | | 2 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 1 | 2 | 2 | 2 | — | 2 | 3 | 3 | — | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| B-E10 | (CD40) | 3 | 1 | 2 | 3 | 3 | 0 | 2 | 2 | 2 | 2 | 2 | 3 | — | 2 | 2 | 3 | — | 2 | 2 | 4 | 0 | 0 | 0 | 0 |
| A6 | (CD45R0) | 2 | 2 | 2 | 2 | 0 | 4 | 2 | 0 | 1 | 2 | 2 | 3 | — | 3 | 2 | 3 | — | 3 | 3 | 3 | 1 | 1 | 1 | 1 |
| 7F7 | (CD54) | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 2 | 4 | 4 | — | 3 | 3 | 3 | — | 2 | 2 | 3 | 0 | 0 | 0 | 0 |
| OKT27 | (CD54) | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 2 | 3 | 3 | — | 3 | 3 | 3 | — | 2 | 2 | 3 | 0 | 0 | 0 | 0 |
| HD66 | (CD76) | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | 1 | 1 | — | 1 | 1 | 2 | 0 | 0 | 0 | 0 |
| B-C5 | | 4 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | 0 | 0 | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22.2 | | 2 | 2 | 3 | 2 | 0 | 1 | 0 | 1 | 1 | 2 | 2 | 1 | — | 2 | 2 | 2 | — | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| ID11 | | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| ACT35 | | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |

PMN, polymorphonuclear neutrophilic leucocytes; 0 = negative; 1 = individual cells positive; 2 = < 50% of the cells positive; 3 = > 50% of the cells positive; 4 = > 90% of the cells positive; — = not evaluated.

Control = Patient O.M.; Pat. 1 = patient A.T.; Pat. 2 = patient G.E.; Pat. 3 = patient M.T.

...n PB ...r SF, a substantially higher expression of ...many ...f the analysed markers could be observed ...n T cells originating from ST. In particular, this ...as the case with M Abs recognizing CD39, ...D40 and CD76, and also for the molecules ...dentified by MoAbs 22.2 and 1D11. Although ...ne results must be viewed with caution since they ...ere obtained only in a single case, they suggest ...hat many activation antigens are up-regulated ...n T cells which enter the inflamed tissue compartment.

*Topographical distribution of activation antigens in the synovium*

In order to examine the distribution of the activation antigens detected by our 12 selected workshop MoAbs on the various cell types present in the ST of RA patients, immunohistological studies were performed. The staining patterns obtained are summarized in Table III. MoAb UCHT1 was chosen as the T-cell marker since it gave optimal results. MoAb UCHT1 detected individual T cells in the synovial lining layer of the control and RA material, as well as individual spindle-shaped cells, a greater number of cells in the inflammatory infiltrates, and almost all lymphoid follicle cells in RA. The classical T-cell activation antigens interleukin-2 receptor (CD25), transferrin receptor (CD71) and MHC class II showed a very divergent distribution. While CD25 was seen only on individual cells in RA inflammatory infiltrates and lymphoid follicles, the other two markers were found in a variety of cells not restricted to CD3⁺ regions. Out of the selected 12 MoAbs, OKT28 and A1 (CD39) stained almost all endothelial cells on control and RA synovium and less than 50% of synovial lining, spindle-shaped and lymphoid follicle cells in RA. MoAb TU66 was negative on RA synovial lining cells and only stained individual cells in RA lymphoid follicles. The CD40 antigen displayed a very broad distribution on nearly all cell types present in RA ST, and about 50% of cells in regions rich in T cells were positive. MoAb A6 (CD45 R0) was found on a majority of cells in lymphoid follicles and inflammatory infiltrates and also in lower frequency on cells in other regions. The antigen CD54 (ICAM-1) was expressed on a large number of synovial lining and endothelial cells in both control and RA synovium, and in addition on approximately 50% of cells in areas rich in T cells. Thus, the results obtained by immunohistology were generally in good agreement with the flow cytometry

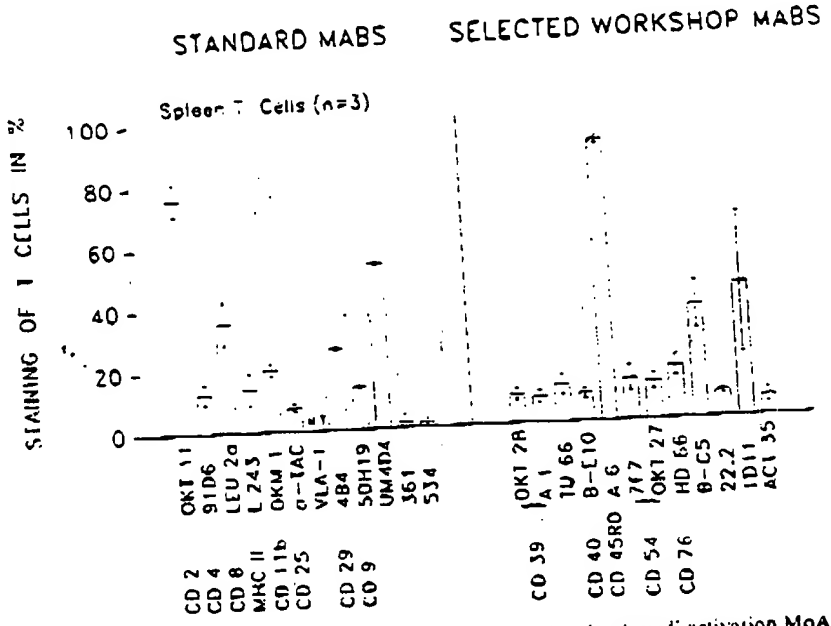


FIG. 4. Staining of spleen T cells with a panel of 'standard' and 'selected' activation MoAbs. Vertical columns represent the mean percentages of staining of CD3⁺ cells ± one standard deviation.

PMN, polymorphonuclear neutrophilic leucocytes; 0 = negative; 1 = individual cells positive; 2 = < 50% of the cells positive; 3 = > 50% of the cells positive; 4 = > 90% of the cells positive; - = not evaluated.
Control = Patient O.M.; Pat. 1 = patient A.T.; Pat. 2 = patient G.F.; Pat. 3 = patient M.T.

data. Exceptions to this rule were the staining pattern with M Abs B-C5 and HD66, the most probable explanation being a failure of the MoAbs to properly bind to denatured antigen. MoAbs 1D11 and ACT35 could not be evaluated since they altogether failed to stain fixed ST in a specific manner.

*Expression of activation antigens on spleen T cells*

There remained the possibility that some surface markers found on SF T cells were acquired as a consequence of a migration through a tissue compartment, and not as a result f a change in cell function induced by inflammation. Since there are no ideal contr ls for T cells taken from inflamed tissue, we analysed the expressi n of the cell surface anugens on T cells removed from spleens. The results are shown in Fig. 4. In comparison with PB T cells several aspects were remarkable: the expression of MHC class II products was similarly low, but there was a clear up-regulation of the antigens CD45 R0 and 1D11, and to a lesser degree of B-C5. Still, in comparison with SF T cells, the levels of staining for ICAM-1, B-C5 and HD66 were substantially lower on spleen T cells.

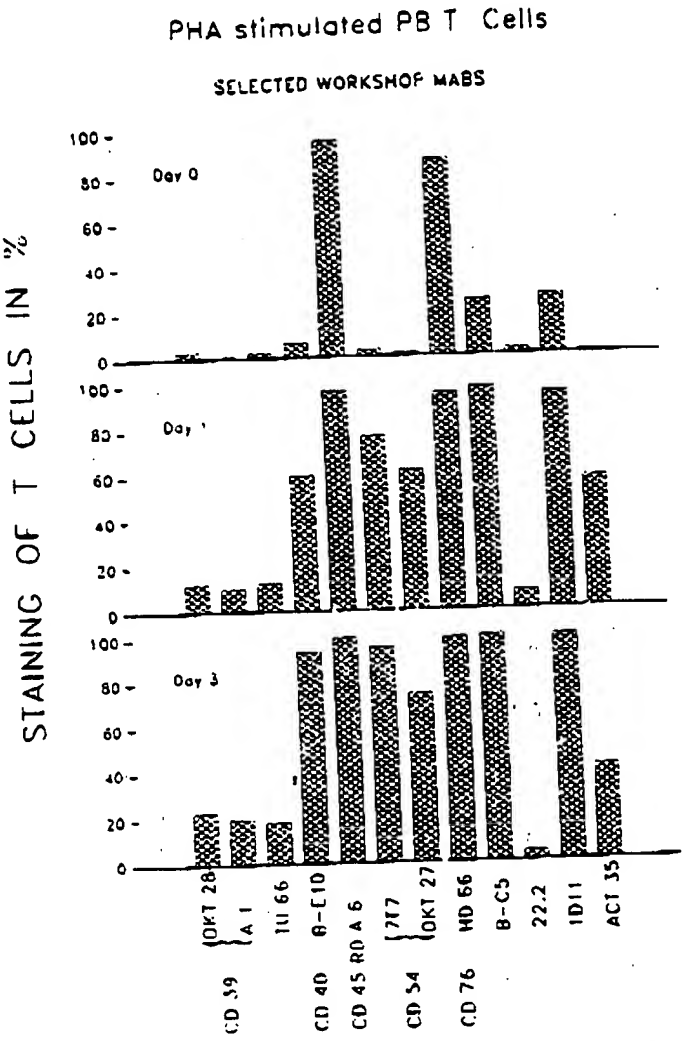


FIG. 5. Expression of activation antigens on PHA-activated T cells on day 0, day 1 and day 3 using two-colour flow cytometry. Vertical columns represent the percentages of staining of $CD3^+$ cells.

*Expression of activation antigens on in vitro activated T cells*

In order to document the degree of expression of the examined activation antigens on fully activated T cells, we stimulated PB mononuclear cells of a donor with PHA and analysed the T cells by two-colour flow cytometry (Fig. 5). This was performed with cells of only one individual, since the results obtained were in perfect accordance with the data collected in a large number of identical experiments [13]. The only deviations were increased percentages for CD45 RO (95% versus 65%) and CD76 (86% versus 35%) on day 0. The data demonstrated that PHA-stimulated T cells express the majority of the examined activation antigens to almost 100% shortly after activation. The only exception were MoAbs recognizing the CD39 and ACT35 antigens, where the levels of staining were only moderately increased. MoAb 22.2 turned out not to recognize a T-cell antigen [13].

## DISCUSSION

The aim of our study was to identify differences in cell surface markers between T cells taken from the PB of healthy individuals and T cells recovered from inflamed joints of RA patients. The basic idea was that an altered functional state of T cells located at the site of inflammation should result in an altered expression pattern of cell surface markers. A precedent is the increased expression of MHC class II molecules on T cells in SF and ST [7, 10], and also to a lesser degree on T cells in the PB [12, 23] of RA patients.

We have screened a great number of MoAbs directed against activation molecules on cells of the haematopoietic lineage for an increased staining of RA T cells. As a result several surface markers were identified which fulfilled this criterion. The most impressive increases in expression on T cells from RA patients were seen with MoAb B-C5, which recognizes an as yet unclustered cell surface molecule, and with MoAb A6, which is specific for the CD45 RO antigen. In addition, we observed elevated levels of expression for ICAM-1 (CD54) as well as for the CD39 and the CD40 molecules. Three other antigens (CD76, 1D11 and ACT35) were present on a substantial number of T cells in some but not all RA patients. The expression of ICAM-1 on SF T cells agrees well with a recent report on the presence of this antigen on lymphocytes in ST sections of RA patients [16]. Similar to our findings, Pitzalis *et al.* [31] described a high expression of CD45 RO, a 180-kDa isoform of the leucocyte common antigen, on T cells from SF of RA patients. The other antigens examined in this study have only been analysed in vitro. The CD39 and CD40 molecules are best characterized on B cells, where they can act as co-stimulatory molecules [14, 35]. The MoAb HD66 is specific for the CD76 antigen; B-C5, ACT35 and 1D11 represent new, unclustered antigens.

A phenomenon common to all of the activation antigens analysed in this study was the hierarchy of expression on T cells from various compartments. Characteristically, the levels of expression were lowest on PB T cells from healthy individuals, already elevated on T cells from the PB of RA patients, and reached the highest values on T cells recovered from SF. An isolated observation made with T cells obtained at the same time from the SF and ST of a single RA patient suggested that even higher levels of activation antigen expression can be found on T cells originating from inflamed ST. This compartmental hierarchy of expression on T cells in RA patients, already observed with MHC class II ([7, 12, 37] and also this study), could be interpreted as a sign of increased cell traffic between inflamed tissue and PB. Any alternative explanation would have to implicate factors with systemic effects on T cells not directly involved in the inflammatory process.

The function of most of the examined antigens is unknown. Nevertheless, some indirect information regarding the role of these antigens in vivo can be gained by the analysis of their tissue distribution. Our results obtained with two-colour flow cytometry already revealed that none of the antigens is restricted to T cells (data not shown), and this was confirmed by immunohistological analysis. Our finding of broad tissue distribution of the examined antigens in vivo is supported by in vitro data collected recently in the activation antigens workshop of the Fourth International Conference on Human Leucocyte Differentiation Antigens. These results, obtained independently in many laboratories, demonstrated that the CD39, CD40, CD45 RO, CD54, CD76, B-C5 and 1D11 antigens are expressed to a high degree on unstimulated B cells as well as on in vitro-activated T cells and monocytes [13]. In these studies only MoAb 22.2 preferentially

stained monocytes, whereas reactivity with MoAb ACT35 was strongly biased towards T lymphocytes. The combined analysis of our in vivo data and the worksh p in vitr results revealed two major features common to the activation antigens selected by our screening protocol: a broad tissue distribution and an up-regulated expression on T cells resident in inflamed tissue or activated in vitro. These expression characteristics could reflect a common functional role as homing receptors and/or molecules mediating cell–cell or cell–matrix interactions. Only the antigens defined by MoAb 22.2 and ACT35 are probable exceptions in such a hypothetical concept due to their rather restricted tissue distribution. The ICAM-1 (CD54) antigen is indeed a well-characterized cell adhesion molecule and its ligand is identified to be LFA-1 [28]. Data obtained for CD45 R0 [30] and CD40 [26] in vivo are compatible with a participation in cell adhesion.

Molecules which determine the potential of cell–cell or cell–matrix interactions could already be expressed on T cells resident in healthy tissue. Since our studies performed on T cells from inflamed joints did not allow a distinction between an antigen up-regulation caused by inflammation and a physiological up-regulation initiated by contact with tissue. T cells resident in normal spleens were also investigated. These data clearly indicated that a strongly elevated expression of the CD45 R0 and 1D11 antigens does not represent a specific response to inflammation, but rather reflects more general functions of these molecules on T cells. In contrast, MHC class II products and several activation molecules from our panel (CD54, CD76, B-C5), which were substantially up-regulated on SF T cells, were present on spleen T cells to a similar degree as on PB T lymphocytes. The increased expression of these antigens thus might be related to an altered functional state of T cells participating in an inflammatory process. The CD39 and CD40 antigens could not be clearly assigned to any of the two groups since the differences in staining between the various compartments were too small. This tentative classification into 'resident' and 'inflammatory' cell surface molecules on T cells has certainly to be validated by analysing a large number of samples from RA patients and patients with other chronic inflammatory diseases.

In conclusion, we have identified a number of cell surface molecules—B-C5, CD39, CD40, CD45 R0, CD54, CD76 and potentially 1D11 which are (i) clearly up-regulated n PB and SF cells in RA patients, (ii) not restricted to inflam tissue, (iii) n t restricted to a particular cell ty and (iv) strongly up-regulated on in vitro-ac vated T cells. It is interesting to note that identified molecules resemble in all these aspe MHC class II products, whose increased pression on a variety of cells, including T cells, clearly implicated in a variety of autoimmu diseases.

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## REFERENCES

1 Abrahamsen, T.G., Frøland, S.S., Natvig, J.I Pahle, J. Elution and characterization of lymp cytes from rheumatoid arthritis inflammatory sue. *Scand. J. Immunol.* 4, 823, 1975.

2 Arnett, F.C., Edworthy, S.M., Bloch, D McShane, D.J., Fries, J.F., Cooper, N.S., Hea L.A., Kaplan, S.R., Liang, M.H., Luthra, H Medsger, T.A., Mitchell, D.M., Neustadt, D Pinalis, R.S., Schaller, J.G., Sharp, J.T., Wil R.L. & Hunder, G.G. The American Rheuma Association 1987 revised criteria for the classi tion of rheumatoid arthritis. *Arthritis Rheum* 315, 1988.

3 Bankhurst, A.D., Husby, G. & Williams, Predominance of T cells ian the lymphocytic trates of synovial tissues in rheumatoid arth. *Arthritis Rheum.* 19, 555, 1976.

4 Beverley, P.C.L. & Callard, R.E. Distinctive f tional characteristics of human "T" lymphoc defined by E rosetting or a monoclonal anti-T antibody. *Eur. J. Immunol.* 11, 329, 1981.

5 Breard, J., Reinherz, E.L., Kung, P.C., Golds G. & Schlossman, S.F. A monoclonal antii reactive with human peripheral blood monoc *J. Immunol.* 124, 1943, 1980.

6 Burmester, G.R., Jahn, B., Gramatzki, M., Zac J. & Kalden, J.R. Activated T cells *in vivo* ar *vitro*: divergence in expression of Tac and Ia gens in the nonblastoid small T cells of inflam tion and normal T cells activated *in vitro Immunol.* 133, 1230, 1984.

Burmester, G.R., Yu, D.T.Y., Irani, A.-M., Kunkel, H.G. & Winchester, R.J. Ia+ T cells in synovial fluid and tissues of patients with rheumatoid arthritis. *Arthritis Rheum.* 24, 1370, 1981.

Cordell, J.L., Falini, B., Erber, W.N., Gosh, A.K., Abdulaziz, Z., McDonald, S., Pulford, K.A.F., Stein, H. & Mason, D.Y. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* 32, 219, 1984.

Evans, R.L., Wall, D.W., Platsoucas, C.D., Siegal, F.P., Fikrig, S.M., Testa, C.M. & Good, R.A. Thymus-dependent membrane antigens in man: inhibition of cell-mediated lympholysis by monoclonal antibodies to $T_{H2}$ antigen. *Proc. Natl. Acad. Sci. USA* 78, 544, 1981.

Forre, O., Doubloug, J.H. & Natvig, J.B. Augmented numbers of HLA-DR positive T lymphocytes in the synovial fluid and synovial tissue of patients with rheumatoid arthritis and juvenile rheumatoid arthritis. *Scand. J. Immunol.* 15, 227, 1982.

Fourth International Conference on Human Leucocyte Differentiation Antigens. *Tissue Antigens* 33, 305, 1989.

Fox, R.I., Fong, S., Sabharwal, N., Carstens, S.A., Kung, P.C. & Vaughan, J.H. Synovial fluid lymphocytes differ from peripheral blood lymphocytes in patients with rheumatoid arthritis. *J. Immunol.* 128, 351, 1982.

Gilks, W. Provisional database of the Fourth International Conference on Human Leucocyte Differentiation Antigens. Vienna, February 1989.

Gordon, J., Millsum, M.J., Guy, G.R. & Ledbetter, J.A. Resting B lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. A comparison with IL-4-mediated signaling. *J. Immunol.* 140, 1425, 1988.

Goto, M., Miyamoto, T., & Nishioka, K. 2 dimensional flow cytometric analysis of activation antigens expressed on the synovial fluid T cells in rheumatoid arthritis. *J. Rheumatol.* 14, 230, 1987.

Hale, L.P., Martin, M.E., McCollum, D.E., Nunley, J.A., Springer, T.A., Singer, K.H. & Haynes, B.F. Immunohistologic analysis of the distribution of cell adhesion molecules within the inflammatory synovial microenvironment. *Arthritis Rheum.* 32, 22, 1989.

Hansen, J.A., Martin, P.J., Beatty, P.G., Clark, E.A. & Ledbetter, J.A. Human T lymphocyte cell surface molecules defined by the workshop monoclonal antibodies ("T cell protocol"). Pp. 195-212 in Bernard, A., Boumsell, L., Dausset, J., Milstein, C. & Schlossman, S.F. (eds) *Leucocyte Typing*. Springer, Berlin, 1984.

Hemler, M.E., Jacobson, J.G., Brenner, M.B., Mann, D. & Strominger, J.L. VLA-1: a T-cell surface antigen which defines a novel late stage of human T cell activation. *Eur. J. Immunol.* 15, 502, 1985.

Higgs, J.B., Zeldes, W., Kozarsky, K., Schteingart, M., Kan, L., Bohlke, P., Krieger, K., Davis, W. & Fox, D.A. A novel pathway of human T lymphocyte activation. Identification by a monoclonal antibody generated against a rheumatoid synovial T cell line. *J. Immunol.* 140, 3758, 1988.

Holter, W., Majdic, O., Liszka, K., Stockinger, H. & Knapp, W. Kinetics of activation antigen expression by *in vitro*-stimulated human T lymphocytes. *Cell. Immunol.* 90, 322, 1985.

Hovdenes, J., Gaudernack G., Kvien, T.K., Egeland, T. & Mellbye, O.J. A functional study of purified $CD4^+$ and $CD8^+$ cells isolated from synovial fluid of patients with rheumatoid arthritis and other arthritides. *Scand. J. Immunol.* 29, 641, 1989.

Konttinen, Y., Bergroth, V. & Nykänen, P. Lymphocyte activation in rheumatoid arthritis synovial fluid in vivo. *Scand. J. Immunol.* 22, 503, 1985.

Kluin-Nelemans, H.C., van der Linden, J.A., Gmeling Meyling, F.H.J. & Schuurman, H.-J. HLA-DR positive T lymphocytes in blood and synovial fluid in rheumatoid arthritis. *J. Rheumatol.* 11, 272, 1984.

Lampson, L.A. & Levy, R. Two populations of Ia-like molecules on a human B cell line. *J. Immunol.* 125, 293, 1980.

Ledbetter, J.A., Evans, R.L., Lipinski, M., Cunningham-Rundles, C., Good, R.A. & Herzenberg, L.A. Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and man. *J. Exp. Med.* 153, 310, 1981.

Ling, N.R., MacLennan, I.C.M. & Mason, D.Y. B-cell antigens: new and previously defined clusters. Pp. 302-336 in McMichael, A.J. (ed.) *Leucocyte Typing III*. Oxford University Press, Oxford, 1987.

MacLean, G.D., Seehafer, J., Shaw, A.R.E., Kieran, M.W. & Longenecker, B.M. Antigenic heterogeneity of human colorectal cancer cell lines analyzed by a panel of monoclonal antibodies. I. Heterogeneous expression of Ia-like and HLA-like determinants. *J. Nat. Cancer Inst.* 69, 357, 1982.

Makgoba, M.W., Sanders, M.E., Ginther Luce, G.E., Dustin, M.L., Springer, T.A. Clark, E.A., Mannoni, P. & Shaw, S. ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature* 331, 86, 1988.

Morimoto, C., Letvin, N.L., Boyd, A.W., Hagan, M., Brown, H.M., Kornacki, M.M. & Schlossman, S.F. The isolation and characterization of the human helper inducer T cell subset. *J. Immunol.* 134, 3762, 1985.

Pitzalis, C., Kingsley, G., Haskard, D. & Panayi, G. The preferential accumulation of helper-inducer T lymphocytes in inflammatory lesions: evidence for regulation by selective endothelial and homotypic adhesion. *Eur. J. Immunol.* 18, 1397, 1988.

Pitzalis, C., Kingsley, G., Murphy, J. & Panayi, G. Abnormal distribution of the helper-inducer and suppressor-inducer T-lymphocyte subsets in the rheumatoid joint. *Clin. Immunol. Immunopathol.* 45, 252, 1987.

Reinherz, E.L. & Schlossman, S.F. The differentiation and function of human T lymphocytes. *Cell* 19, 821, 1980.

Sasaki, D.T., Dumas, S.E. & Engleman, E.G. Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. *Cytometry* 8, 413, 1987.

Uchiyama, T., Broder, S. & Waldmann, T.A. A

monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J. Immunol.* 126, 1393, 1981.

35 Valentine, M.A., Clark, E.A., Shu, G.L., Norris, N.A. & Ledbetter, J.A. Antibody to a novel 95-kDa surface glycoprotein on human B cells induces calcium mobilization and B cell activation. *J. Immunol.* 140, 4071, 1988.

36 Verbi, W., Greaves, M.F., Schneider, C., Koubek, K., Janossy, G., Stein, H., Kung, P. & Goldstein, G. Monoclonal antibodies KT1 and OKT11A have pan-T reactivity and block sheep erythrocyte "receptors". *Eur. J. Immunol.* 12, 81, 1982.

37 Waalen, K., Førre, Ø., Linker-Israeli, M. & Thoen, J. Evidence of an activated T-cell system with augmented turnover of interleukin 2 in rheumatoid arthritis. Stimulation of human T lymphocytes by dendritic cells as a model for rheumatoid T-cell activation. *Scand. J. Immunol.* 25, 367, 1987.

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February 23, 1996

Ms. Helen Williams
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Re: The manuscript by Genhong Cheng, Aileen M. Cleary, Zheng-sheng Ye, David I. Hong, Seth Lederman, and David Baltimore entitled Involvement of CRAF1, a Relative of TRAF, in CD40 Signaling, Science (March 10, 1995) 267(5203):1494- 1498

Dear Ms. Williams:

I understand from our February 22, 1996 telephone conference that the subject manuscript entitled "Involvement of CRAF1, a Relative of TRAF, in CD40 Signaling" was published in the March 10, 1995 issue of Science which was mailed on March 10, 1995.

If the foregoing is accurate, please sign and date the enclosed copy of this letter where indicated and return the signed copy to me in the enclosed, prepaid, self-addressed envelope.

Thank you for your assistance.

Sincerely,

Lewis J. Kreisler

Lewis J. Kreisler

LJK/lnb
Enclosure

This is to confirm that the manuscript by Genhong Cheng, Aileen M. Cleary, Zheng-sheng Ye, David I. Hong, Seth Lederman, and David Baltimore, entitled Involvement of CRAF1, a Relative of TRAF, in CD40 Signaling was mailed on March 10, 1995.

2/29/96
Date

Helen C. Williams
Helen Williams